INTERACTION OF AZO DYES AND RAT LIVER PREPARATIONS

by

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TABLE OF CONTENTS

INTRODUCTION

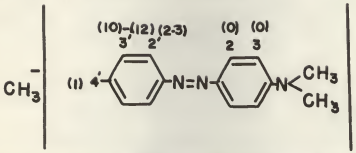
A tumor or neoplasm may be defined as a growth of new cells which proliferate without control and which serve no useful function. If one can study such growth and learn of its nature then one may predict and control its formation.

Although the oncologist has an extensive classification of tumors and carcinogenic agents, comparatively little is known concerning the relationship of the carcinogenic agent to the tumor formation or cancer. Growth and development, both normal and abnormal, are concerned with protein synthesis. It would seem reasonable then to center our attention around these human building blocks.

Listed among the carcinogenic agents, of the oncologist, are certain chemical compounds. These compounds have become useful instruments for the study of the interaction of carcinogenic agents and tumor formation. Although the extent of the reaction and the type of tumor resulting may not be the same for each chemical compound, it seems reasonable to assume that essentially the same biochemical changes will take place regardless of the specific character of the chemical compound. On the basis of such a hypotheses an extensive investigation of chemical carcinogens has been carried out in the past twenty years.

Among the chemical carcinogens are certain aminoazo dyes. In 1937 Kinosita (4) reported that 4-dimethylaminoazobenzene (DAB) was an active hepatic carcinogen for rats. This dye and

certain of its derivatives have become the most widely employed compounds in the study of aminoazo dye carcinogeneses. Miller and Baumann (10) in 1945 observed that the substitution of methyl groups into the aromatic ring affected its carcinogenicity. The parent compound DAB is assigned an activity of six and the relative activities of the methyl derivatives are shown in parenthesis above the ring position numbers.



Thus 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) is a stronger carcinogen than 4-dimethylaminoazobenzene (DAB); which in turn is stronger than 2'-methyl-4-dimethylaminoazobenzene (2'-Me-DAB); which in turn is stronger than 4'-methyl-4-dimethylaminoazobenzene (4'-Me-DAB).

The Millers (13) proposed the theory that the aminoazo dyes, e.g. 3'-Me-DAB, initiate the carcinogenic process through the formation of chemical linkages to certain of the liver proteins. The bound dyes are found only in the liver, the site of tumor formation. Price et al. (18) also found that the bound dyes were not found in the liver tumor. This would seem to indicate that the tumor and normal tissue each have a certain specific character in their relative affinity for the dyes. This dye-protein complex cannot be destroyed by prolonged extraction

with organic solvents, or by dialysis. The dyes are released upon destruction of the liver protein molecules with enzymes (in vitro), acid hydrolysis, or strong alcoholic alkali.

Miller et al. (11) contend that the binding of the dyes to the protein takes place at the $-N(CH_3)_2$ group or possibly to the ring to which this group is attached. Later work by the Millers indicates that the N-methyl dyes are converted to N-methylol dyes which appear to be the active intermediates in the formation of the protein-bound dyes (12). Using 3'-Me-DAB labeled with C^{14} in the 3'-methyl position, radioactivity has been found in the riboflavin fraction of rat liver (20).

A larger portion of the dyes is bound by the water soluble proteins of the liver than by any other fraction (18) indicating the importance of these proteins in the neoplastic transformation. Further indication that changes occur in the water soluble proteins involving 3'-Me-DAB was found by Griffin et al. (2). The globulin fraction was doubled and there was a slight decrease in the albumin in the precancerous state. It was shown also that at the end of eight weeks there was marked rise in the amount of desoxyribonucleoprotein (DNP), a slight decrease in the ribonucleoprotein (RNP), but no significant change in the albumin fraction. Investigators (22) have reported that the concentration of nucleic acid in tumor-bearing animals is greater than in normal animals, although nucleic acids do not bind with aminoazo dyes. The workers at Wisconsin (9) claim that liver tumors have a higher nuclear density than normal

liver and therefore contain more desoxyribonucleic acid per gram of tissue than ordinary liver. Kehler and Robertson (3) found a higher acidity in cancer liver tissue (pH 6.99), than in normal liver (pH 7.39). The increased nucleic acid content of the malignant tissue is perhaps the most likely explanation of this difference. Relatively little is known concerning the liver protein during aminoazo dye carcinogenesis.

Assuming that the aminoazo dyes are chemically bound to rat liver by the N-methyl group, and that there is a structural relationship between the rate of uptake of the dye (13) (11) and carcinogenicity it was thought desirable to conduct a study of the homologs of DAB with normal and tumor rat liver. This study would give a comparison of the rate of uptake of each homolog of DAB with tumor and normal liver and show also the constituent of the rat liver that was doing the binding.

The dyes chosen for this experiment were 4-aminoazobenzene, 3'-methyl-4-aminoazobenzene, 4'-methyl-4-aminoazobenzene and methyl orange. The first three dyes are homologs of the carcinogens 4-dimethylaminoazobenzene, 3'-methyl-4-dimethylaminoazobenzene and 4'-methyl-4-dimethylaminoazobenzene respectively. The homolog of 2'-methyl-4-dimethylaminoazobenzene was not used due to difficulty in synthesis of the dye. These dyes were chosen rather than their dimethyl homologs because the latter series of compounds were not sufficiently soluble in water to allow accurate analytical determinations.

Table 1. Solubilities of homologs of dimethylaminoazobenzene (16).

Dye	Solubility moles/liter pH 6.8 25° C.
4-aminoazobenzene	1.388×10^{-4}
3'-methyl-4-aminoazobenzene	1.788×10^{-5}
4'-methyl-4-aminoazobenzene	1.592×10^{-5}

Methyl orange, an anionic dye, was only used for comparative purposes in the study of normal and tumor homogenates and fractions. The homologs of DAB are uncharged dyes. It was hoped that the results obtained from the aminoazobenzene series would be applicable to the dimethylaminoazobenzene series because of the analogous structural relationship of the two series of dyes.

The study of the interaction of the dyes with the rat liver was broken into two main divisions:

(1) Normal and tumor liver fractions.

(2) Normal and tumor liver homogenates.

The normal and tumor liver fractions studied were albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein.

Because of the relative importance of the water soluble fraction, it was hoped that this soluble fraction would be characteristic of the liver as a whole. An investigation was carried out therefore to determine the relative affinity of the soluble normal liver homogenate with uncharged aminoazo dyes and a similar study of the interaction of uncharged aminoazo dyes with soluble tumor liver homogenate.

Configurational differences between the respective proteins might be obtained through binding with organic dyes. It was suggested that this might apply also to the rat liver homogenates. Spectral changes were used to indicate qualitative binding and the dialysis equilibrium method, as developed by Klotz (5) and his co-workers, was used for a more quantitative determination of the degree of binding.

The dyes have a highly specific absorption spectrum. The change in absorption spectrum of a dye, when mixed with protein, is a reflection of the extent and nature of the binding to the fraction (6).

Evidence (7) seems to indicate that the cationic centers on the protein molecule are involved in the binding process in the case of methyl orange, whereas a phenolic tyrosine residue would be involved in the binding process of the uncharged dyes. Much work has been done with cationic and anionic dyes. It is quite conceivable that if two proteins, for example, a normal and a tumor liver, produce a remarkably different absorption spectra with the same anionic dye under essentially identical conditions, there must be at least a difference in configuration around the cationic loci on the protein molecule or at least one can assume that the arrangements of amino acid residues are different.

BASAL RATION

Two groups of Sprague-Dawley male albino rats, 180-200 grams in weight, were used in this experiment. To the first

group, which shall be known as "normal", the basal ration was fed ad libitum. To the second group, which shall be known as "tumor", the ration was incorporated with 0.06 per cent by weight of 3'-Me-DAB and fed ad libitum. Giese and Miller (1) reported that for an equivalent concentration of dye, rats fed 3'-Me-DAB invariably lost more weight, developed a more severe cirrhosis and formed large hepatic tumors more rapidly than when any other compound was fed. All rats were given one drop of halibut liver oil orally each month. The feeding was carried out for a fourteen week period.

The basal diet was essentially the same as that of the Wisconsin Group (19) and consisted of the following:

Casein (vitamin free)	12%
Glucose	79%
Corn Oil (Mazola)	5%
Salts Mixture	4%

The supplements added per kilogram of ration were:

Thiamine Chloride	3.0 mg
Riboflavin	2.0 mg
Pyridoxine Hydrochloride	2.5 mg
Calcium Pantothenate	7.0 mg
Choline Chloride	30.0 mg

The salts mixture was composed of:

	Parts by Weight
NaCl	1470
Ca_3PO_4	2086
$MgSO_4\ 7H_2O$	558

KCl	1680
$CaCO_3$	2940
$FePO_4$	140
KH_2PO_4	4340
$MgCO_3$	672
KI	1.2
$MnSO_4\ H_2O$	3.2
$K_2Al_2(SO_4)_2\ 24H_2O$	1.2
NaF	7.4
$CuSO_4\ 5H_2O$	5.4

The average amount of ration consumed daily per rat was about 15.0 grams.

Factors in the ration that have been reported (14) to retard tumor development include liver, yeast, grain, the combination of protein and the "B" vitamins and the combination of cysteine and choline. It appears to be well established that the incidence of tumors caused by feeding 3'-Me-DAB depends upon the character of the diet during the precancerous period.

LIVER PERFUSION

Since aminoazo dyes bind with blood it was necessary to have a blood free liver fraction and homogenate. The animals were anesthetized with ether and an incision made to expose the chest and abdominal cavities. The inferior vena cava was

then freed and a thread passed under the vessel. A thirteen gauge hypodermic needle was inserted into the vessel and the thread tied to keep the vessel from retracting. The portal vein was then severed and the needle directed to each lobe of the liver allowing the 0.89 per cent saline to flow through the liver and out the portal vein. When the liver was completely blanched the perfusion was stopped, the liver excised and quick-frozen in a vessel surrounded by an acetone-dry ice bath. The livers were stored for about a week in the freezing compartment of a refrigerator and then homogenized from five to ten minutes at 0° C in an ice-jacketed Waring Blendor. The homogenate was then dialyzed against seven or eight changes of distilled water for 24 to 36 hours, in a cold room at about 4° C, to remove any traces of colored materials not bound to the liver proteins. The homogenates were then lyophylized. This gave a dry homogeneous preparation. The livers used for the fractionation were prepared in a similar manner.

LIVER FRACTIONATION

After the livers were perfused and stored in the freezing compartment of a refrigerator for five to seven days they were fractionated into four protein components (albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein) on the basis of differential solubility in solutions of sodium chloride and ammonium sulfate.

The livers from five to seven rats usually constituted one group for the fractionation procedure. The procedure, originated by Mirsky and Pollister (15) with modifications by Griffin et al. (2), was as follows.

A mixture of the liver and 3.5 parts by weight of neutral 0.4 M sodium chloride was homogenized for two minutes in a Waring Blendor. The entire fractionation procedure was carried out in a room maintained at 0° to 4° C. After ten to fifteen minutes of stirring, the homogenate was centrifuged for fifteen minutes at 3200 RPM (International Centrifuge No. 2). The supernatant fluid (A) was removed and the residue again extracted with a volume of 0.4 M sodium chloride equal to that of the above supernatant fluid. Following this extraction the remaining residue was stirred with 1.5 volumes of 1.0 M sodium chloride and again centrifuged for one hour at 3200 RPM. The supernatant fluid (B) was removed and the residue discarded.

Albumin, globulin and ribonucleoprotein were obtained from the initial 0.4 M sodium chloride extracts and the 1.0 M sodium chloride extract (B) contained the desoxyribonucleoprotein.

The extract (A) was reduced to a pH of 5.0, allowed to stand for one hour and then centrifuged for one hour at 3200 RPM.

The residue (C) contained the ribonucleoprotein. The supernatant fluid containing the globulin and albumin was dialyzed overnight with running distilled water. The precipitated globulin was centrifuged off. The further addition of solid ammonium sulfate to 4.6 M (60.8 grams of $(NH_4)_2SO_4$ per 100 ml of supernatant) and adjustment of the pH to 6.5 precipitated the albumin fraction.

The residue (C) was stirred in water and the pH adjusted to 8.0 to dissolve the ribonucleoprotein. Adjusting the pH to 5.0 and repeating the process precipitated a relative pure fraction of ribonucleoprotein.

The desoxyribonucleoprotein was precipitated from the 1.0 M extract (B) by changing the molarity of the sodium chloride to 0.14 M (6.1 ml of water for every ml of 1.0 M NaCl-DNP solution). At this concentration the protein appears in a fibrous state and can be removed with a stirring rod. The fibers were then redissolved in 1.0 M sodium chloride. This procedure, repeated three times resulted in a pure preparation of DNP. Acetic acid and sodium hydroxide were used to adjust the various pHs.

All the proteins were dialyzed for twenty-four hours and then lypholized. This gave a dry pure preparation of the various fractions.

BINDING TECHNIQUE

The buffer used was made up to have a pH value of 6.8. It was prepared by dissolving 7.87 grams of anhydrous disodium hydrogen phosphate and 6.0 grams of anhydrous potassium dihydrogen phosphate in distilled water and diluting to one liter.

The spectra of the dye and the dye liver complex was made as follows. For the spectrum of the dyes, the buffer was placed in one cell for a blank, and the solution of dye in buffer in the other cell. For the dye complexes, 5 ml of a 0.4 per cent

solution of the liver was placed in two test tubes. Twenty ml of the dye solution was placed in one tube and 20 ml of buffer in the other tube. The solutions were shaken for a moment and then read. The buffer-liver solution was a blank for the dye-liver solution and thus the spectrum of the dye-liver complex was obtained.

Beers-Lambert law was used to calculate the concentration.

$$\text{Optical Density} = \log I_o/I = \varepsilon.cl$$

I_o is the intensity of the incident light and I is the intensity of the light transmitted through a distance l of the solution. ε is the molar extinction coefficient, c is the concentration of the dye in mols/liter. The extinction coefficients were provided by Dr. R. K. Burkhard.

The method consisted essentially of immersing a casing bag with a known concentration of the filtered liver homogenate or protein fraction into a solution of the dye under investigation. The tube was placed in a mechanical shaker for sixteen hours for the system to reach equilibrium. The temperature was maintained at 0° C with a crushed ice-water bath. The concentration of the free dye was determined spectrophotometrically on a Beckman Spectrophotometer, model DU, using 5 cm quartz cells. A control, to establish the approximate quantity of free dye which was present in the absence of the liver homogenate or protein fraction, was set up by placing the buffer in the casing bag and putting this in the dye under exactly the same conditions.

DETERMINATION OF LIVER SOLUBILITY

The liver homogenate was not completely soluble in the buffer solution. Since this was the case, it was necessary to determine the degree of solubility of the normal and tumor liver homogenates. A sample of the liver homogenate was weighed and dried to constant weight over phosphorus pentoxide. The homogenate was then diluted with buffer and filtered with a fritted glass filter. The residue was dried to a constant weight over phosphorus pentoxide and the amount of soluble liver homogenate was calculated by difference, the results are shown in Table 2.

PRECIPITATION OF PROTEIN

The protein fraction of both the normal and tumor liver homogenates were precipitated by a method similar to that of Miller and Miller (13). A sample of the liver homogenate was weighed and diluted to 0.4 per cent concentration with phosphate buffer. The solution was filtered and 8.2 grams of sodium acetate was added to each 100 ml of the filtrate. The pH was then adjusted to 5.0 with acetic acid. The solution was then added to a round bottom flask fitted with a reflux condensor and boiled for three minutes. This precipitated the protein. The solution was filtered and the filtrate added to a casing bag and dialyzed for eighteen hours to remove any acetate, which would

interfere with the binding. A ferric chloride spot test was used to show the absence of acetate. The protein free solution was then lypholyzed and tested for binding by using 4'-methyl-4-aminoazobenzene.

After the precipitation of the protein aliquot solutions were treated with trichloroacetic acid and sodium tungstate respectively, but no additional protein could be precipitated.

DETERMINATION OF GLYCOGEN IN LIVER

A comparative method was used to determine the amount of glycogen in the normal and tumor liver homogenates. A sample of pure glycogen was weighed and diluted with phosphate buffer to a known concentration. About 8 mg of oxalic acid and a few drops of dilute sulfuric acid were added to this solution. The mixture was then heated to charing and covered with a small watch glass that carried on its underside a piece of filter paper impregnated with o-dianisidine in glacial acetic acid. The vapors produced turn the reagent paper violet, and indicated carbohydrate. A water soluble sample of the normal and tumor liver homogenates were treated in a similar manner. The absence or presence of color on the reagent paper was an indication of the amount of glycogen in the respective homogenates. The amount of glycogen present in the water soluble fraction of the normal and tumor liver homogenate was less than 0.3 per cent.

DETERMINATION OF LIPID IN LIVER

The determination of lipid material in the water soluble fraction of normal and tumor liver homogenate was made as follows. An extraction thimble was extracted with chloroform in a Soxhlet Extractor for seventy-two hours to remove any lipid material which might be present in the thimble. The thimble was first air dried then dried over phosphorus pentoxide under vacuum to a constant weight. A sample of the liver homogenate was weighed and diluted to 0.4 per cent with phosphate buffer and filtered. The filterate was dialyzed for twenty-four hours to remove the phosphate. The solution was then lypholyzed. A sample of the lypholyzed material was weighed with the lipid free thimble and then extracted for seventy-two hours with chloroform, dried and reextracted and dried until a constant weight was obtained. The amount of lipid in the water soluble fraction of the normal and tumor liver homogenates was then calculated by difference. The amount of lipid present in the normal and tumor water soluble homogenates was negligible.

DETERMINATION OF NONPROTEIN NITROGEN

A nonprotein nitrogen determination was made on both the normal and tumor liver homogenates, prior to and after precipitation. The Micro-Kjedahl technique, essentially the same as described by Sobel et al. (21), was used. The homogenate was digested with equal portions of concentrated sulfuric acid.

The sample was collected in a 0.4 per cent boric acid solution and titrated with 0.0984 N sulfuric acid diluted 1-10 using a mixed indicator (methyl red and methylene blue). The standard, used as a blank, was ammonium sulfate which contained 0.56032 mg/ml of nitrogen. The following ratio was used for the calculation of per cent protein.

$$\frac{\text{ml standard}}{\text{mg standard}} = \frac{\text{ml unknown}}{\text{mg unknown}}$$

$$\text{per cent protein} = 6.25 \times \text{mg unknown nitrogen}$$

The results are shown in Table 3.

RESULTS AND DISCUSSION

Methyl orange and 4-aminoazobenzene did not complex with the normal or tumor liver fractions of albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein. No spectral lowering or shift was observed and the equilibrium dialysis technique gave no positive indications of a protein-dye complex.

This result contradicts earlier observations made in this laboratory. At that time a blood free liver fraction was not used and the binding observed was later attributed to the presence of blood in the various fractions.

It is stated in the introduction that methyl orange, the anionic dye, is attracted by positive loci on the protein molecule and makes the dye-protein complex possible. If the protein molecule is insufficient in positive loci or contains a strong electrostatic repulsive negative charge the dye-protein complex

may not form. This may well be the case with ribonucleoprotein and desoxyribonucleoprotein for they each contain the negatively charged phosphate groups. Perhaps an insufficient number of positive loci on the albumin and globulin fractions would account for the lack of binding with methyl orange. There is, at the present time, no sufficient explanation for the lack of binding of methyl orange and 4-aminoazobenzene to the various protein fractions. It is possible that the protein fractions may have been altered in the fractionation procedure.

Table 2. Solubility of normal and tumor liver homogenates.

Homogenate	Solubility mg/ml
Normal liver homogenate	0.9054
Tumor liver homogenate	0.9580

It is interesting to note the slight difference in the solubilities of the two liver homogenates. Morrione (17) has reported that the concentration of collagen is doubled in cirrhosis due to 4-dimethylaminoazobenzene. Therefore, a decrease in the solubility of the 3'-Me-DAB tumor homogenate would be expected. It was stated in the introduction that the globulin fraction was doubled in the precancerous state and would tend to increase the solubility of the tumor homogenate. The difference in solubility of the two homogenates perhaps is not significant. It probably reflects the entire protein, fat and dye concentrations in the individual animal.

The per cent of protein in the water soluble fractions of the normal and tumor liver homogenates is shown in Table 3.

Table 3. Per cent of protein in the normal and tumor liver homogenates.

Homogenate	Per cent nitrogen x 6.25 : Prior to precipitation	: After precipitation
Normal Liver Homogenate	56.85	9.67
Tumor Liver Homogenate	71.76	9.79

These results seem to indicate that the tumor liver homogenate contains the larger amount of protein. Upon gross examination of the normal and tumor livers it was found, in some cases, that the tumor liver compared with the normal liver has tripled in size. From the solubility data in Table 2 this is not so obvious. But a comparison of ratios the solubility data tends to substantiate the findings as shown in Table 3.

The normal and tumor liver homogenates did not complex with methyl orange. The absorption spectra of methyl orange is shown in Fig. 1. No change in spectra could be observed with either homogenate and the equilibrium dialysis technique did not indicate any degree of binding. Although Klotz (8) found that negatively charged compounds bind with bovine serum albumin it was apparent that there must be a difference in the amino acid residues of rat liver homogenate as compared to bovine serum albumin. This difference could lie in the arrangement, number and kind of different basic amino acid residues of the protein. These differences would then markedly affect the binding of anions such as methyl orange. These results show that anionic dyes, such as methyl orange, do not complex with normal or tumor liver homogenates or their respective protein fractions. Since the amount of glycogen and lipid was not of sufficient

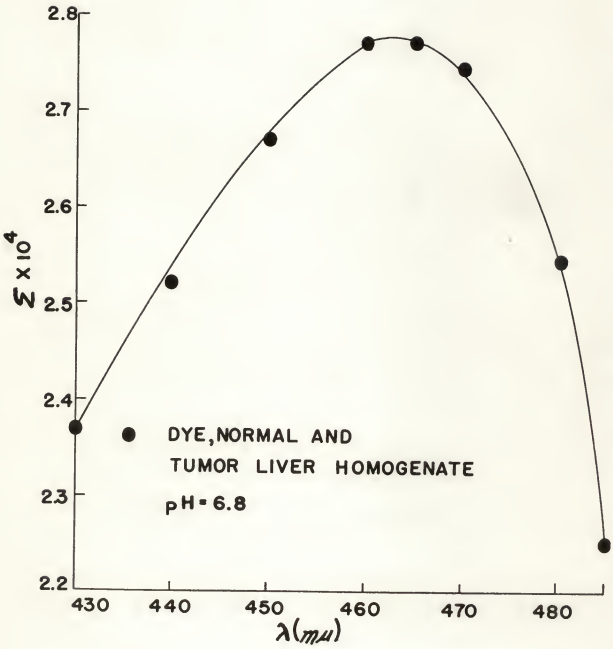


Fig. 1. Spectra of methyl orange and its complexes.

concentration in the liver homogenates to affect the binding in a positive or negative result then the lack of binding of methyl orange could be attributed to the amino acid residue arrangement of the protein fraction of the liver homogenates.

The qualitative examination of the normal and tumor liver homogenates with 4'-methyl-4-aminoazobenzene (Fig.2) indicates that the tumor liver homogenate reacts with the dye to a greater extent than the normal liver homogenate.

Similar results were found in the case of 3'-methyl-4-aminoazobenzene (Fig.3). In this case there appears to be a slight shift of the absorption spectrum to the right for the tumor-dye complex. It is also noted that the normal homogenate compared to the tumor homogenate seems to bind very little.

Fig. 4 shows the absorption spectra of 4-aminoazobenzene. In this case it appears that the normal liver homogenate reacts to a greater extent with the dye than the tumor liver homogenate. This result is just the reverse of that found in the case of 4'-methyl-4-aminoazobenzene and 3'-methyl-4-aminoazobenzene.

In each of the above cases, except for the tumor and 3'-methyl-4-aminoazobenzene complex, there was no shift in the maximum absorption peak of the dye-homogenate complex. But in each case there was a distinct lowering of the maximum absorption. One may assume that the spectral displacements were due to binding since the dyes used at these concentrations obey Beer's Law. Although there was a difference in the absorption spectra of the various dyes with normal and tumor liver homogenates, this was

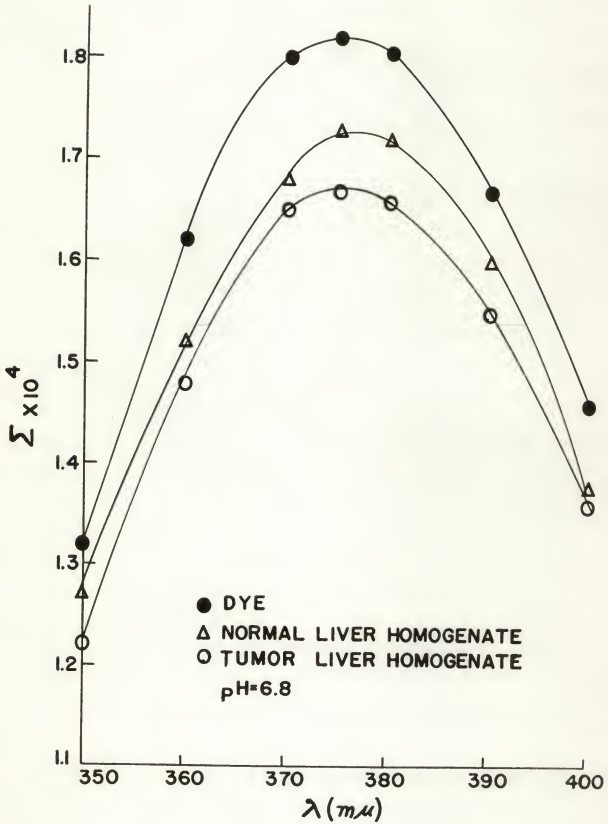


Fig. 2. Spectra of 4'-methyl-4-aminoazobenzene and its complexes.

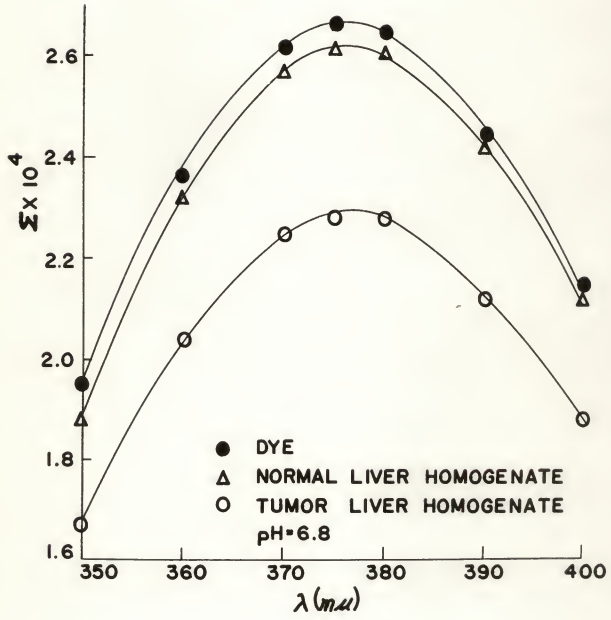


Fig. 3. Spectra of 3'-methyl-4-aminoazobenzene and its complexes.

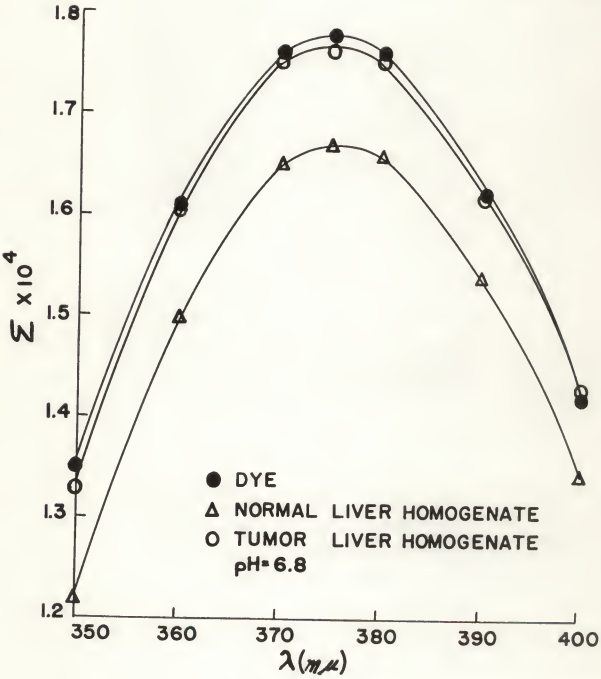


Fig. 4. Spectra of 4-aminoazobenzene and its complexes.

not necessarily conclusive evidence for the behavior of the dye-homogenate complex.

The data obtained from the quantitative measurements of the binding by the equilibrium dialysis method is summarized in Tables 4 and 5 and in Fig. 5.

Table 4. Mean per cent of dye bound per mg of normal liver homogenate.

Dye	Mean per cent of dye bound	Standard deviation
4'-methyl-4-aminoazobenzene	10.32	4.68
3'-methyl-4-aminoazobenzene	6.79	4.20
4-aminoazobenzene	4.33	2.03

Table 5. Mean per cent of dye bound per mg of tumor liver homogenate.

Dye	Mean per cent of dye bound	Standard deviation
4'-methyl-4-aminoazobenzene	13.08	4.56
3'-methyl-4-aminoazobenzene	8.55	2.84
4-aminoazobenzene	4.84	2.58

The major constituents of the water soluble fraction of the normal and tumor liver homogenates are protein, lipid and glycogen. The amount of glycogen in the water soluble fractions has been shown to be less than .3 per cent and the amount of lipid material in the two fractions is also negligible. Therefore, this indicates that the protein fraction of the two homogenates is the only fraction which is actually involved in the formation of the dye-homogenate complex. No investigation has

been carried out to determine the amino acid residues in this protein fraction. It is known, however, that a phenolic tyrosine type of residue is necessary for the formation of omplexes with uncharged dyes. Regardless of the type of protein involved in this dye-protein complex, the comparative degree of interaction of the two homogenates has been obtained. It is apparent that the dyes react with the soluble protein in the same general manner. That is, in both the normal and tumor liver homogenates the 4'-methyl-4-aminoazobenzene is bound to a greater degree than the 3'-methyl-4-aminoazobenzene, and this compound in turn is bound more than 4-aminoazobenzene.

Although the relative basicity of the dyes has not been determined, 4'-methyl-4-aminoazobenzene appears to be the most basic and followed in this respect by 3'-methyl-4-aminoazobenzene and then 4-aminoazobenzene would be the least basic dye. It is interesting to note that the larger per cent of binding is associated with the most basic dye and the smaller per cent of binding is associated with the least basic dye.

There appears to be a relationship between the solubility of the dyes and the per cent of binding of the dyes with the protein fraction of the two homogenates. The least soluble dye, 4'-methyl-4-aminoazobenzene is bound the most to the protein fractions of the two homogenates and the most soluble dye, 4-aminoazobenzene, is bound the least to the two proteins, normal and tumor fractions.

At low concentrations (0.1×10^{-5} to 0.3×10^{-5} M/L) the difference in binding of the methyl isomers was negligible, but as the concentration was increased the difference became more significant. An increase in concentration did not seem to have any appreciable effect upon the binding of 4-aminoazobenzene. At increased concentrations there was a greater difference in the amount of binding of the methyl isomers and 4-aminoazobenzene than there was between the individual methyl isomers.

It was thought originally that the homologs of the carcinogens would bind with the liver protein in the same order as their relative activities as carcinogens. This apparently is not the case. Although the homolog of the weakest carcinogen was bound to the protein in the greatest quantity this does not seem to be significant. It is also apparent that there is no relationship between the solubility of the homologs of the carcinogens and the relative activities of the carcinogens in their ability to produce tumors. However, the homolog of the weakest carcinogen is the least soluble aminoazo dye.

Since the tumor liver homogenate contains the largest amount of protein it should follow that the tumor homogenate should bind more than the normal liver homogenate which contains the smaller amount of protein. This was found to be true. It is also shown that the ratio of per cent of dye bound per cent of protein is similar for each homogenate.

It has not yet been determined conclusively how the uncharged aminoazo dyes react with the liver protein or why the tumor protein binds more than the normal protein. The dye-protein

complex could be made through hydrogen bonding of the HO^- group of a tyrosine residue and the $-NH_2$ group of the dye. Perhaps the molecules are drawn together by van der Waals forces.

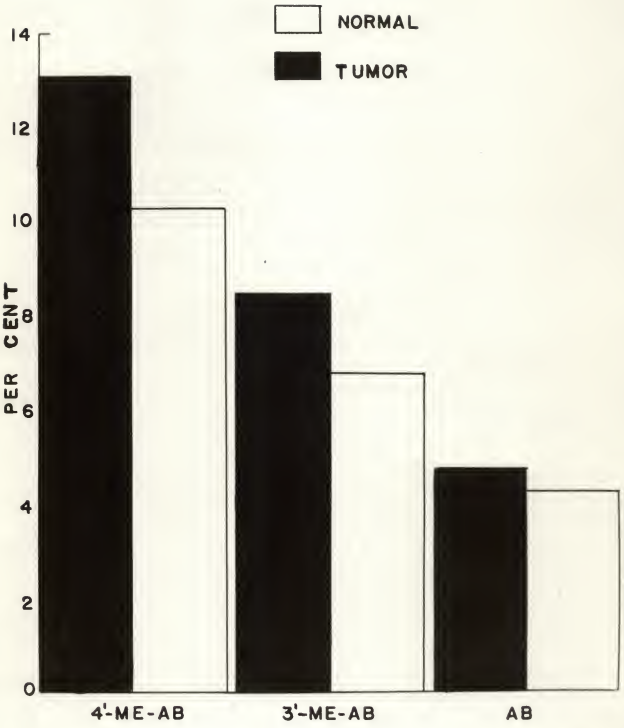


Fig. 5. Per cent of dye bound per mg of liver homogenate.

SUMMARY

The absorption spectra, protein-dye complex and the degree of binding of normal and tumor homogenates, albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein have been studied with uncharged aminoazo dyes. The per cent of protein, glycogen and lipid in the water soluble fractions of the homogenates have been determined. The solubility of the homogenates was also determined. The equilibrium dialysis technique was used to give quantitative evidence concerning the binding of the various fractions.

The per cent of binding and absorption spectra indicates that the normal and tumor liver fractions do not interact with uncharged aminoazo dyes and also indicates that the tumor liver protein binds more with uncharged aminoazo dyes than the normal liver protein.

Methyl orange, an anionic dye, does not interact with the liver homogenates or their fractions.

The amount of lipid and glycogen in the liver homogenates was found to be insignificant and the resulting binding was due to the protein portion of the liver alone.

The tumor liver homogenate was found to be more soluble than the normal liver homogenate.

The methyl substitutions in the aromatic ring alters the solubility, basicity and the extent of interaction of the dye and the protein of the liver homogenate.

It is apparent that there is an increase in the number of functional groups of the tumor protein homogenate or an increased ease in the reactivity of these groups. Hydrogen bonding or van der Waals forces could account for the protein-dye interaction.

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APPENDIX

Spectra of methyl orange and its complexes.

Beckman Spectrophotometer Model DU

Temp. 25° C. pH 6.8

Wavelength m.micron	Dye: Optical Density	Dye: Extenction Coefficient x 10^4	Dye-Normal complex: Optical Density	Dye-Normal complex: Extenction Coefficient x 10^4	Dye-Tumor complex: Optical Density	Dye-Tumor complex: Extenction Coefficient x 10^4
450	.534	2.67	.535	2.67	.535	2.67
460	.554	2.77	.555	2.77	.555	2.77
465	.555	2.77	.555	2.77	.555	2.77
470	.548	2.74	.548	2.74	.548	2.74
480	.508	2.54	.510	2.55	.510	2.55
490	.450	2.25	.448	2.24	.448	2.24
500	.360	1.80	.360	1.80	.360	1.80

Spectra of 4'-methyl-4-aminoazobenzene and its complexes.

Beckman Spectrophotometer Model DU

Temp. 25° C. pH 6.8

Wavelength m.micron	Dye: Optical Density	Dye: Extenction Coefficient x 10^4	Dye-Normal complex: Optical Density	Dye-Normal complex: Extenction Coefficient x 10^4	Dye-Tumor complex: Optical Density	Dye-Tumor complex: Extenction Coefficient x 10^4
350	.265	1.32	.245	1.27	.245	1.22
360	.325	1.62	.305	1.52	.296	1.48
370	.360	1.80	.337	1.68	.330	1.65
375	.365	1.82	.347	1.73	.335	1.67
380	.363	1.81	.344	1.72	.332	1.66
390	.335	1.67	.320	1.60	.310	1.55
400	.293	1.46	.277	1.38	.273	1.36

Spectra of 3'-methyl-4-aminoazobenzene
and its complexes.

Beckman Spectrophotometer Model DU

Temp. 25° C. pH 6.8

Wavelength m.micron	Dye: Optical Density	Dye: Extention Coefficient x 10^4	Dye-Normal complex: Optical Density	Dye-Normal complex: Extention Coefficient x 10^4	Dye-Tumor complex: Optical Density	Dye-Tumor complex: Extention Coefficient x 10^4
350	.390	1.95	.377	1.88	.335	1.67
360	.472	2.36	.464	2.32	.408	2.04
370	.525	2.62	.515	2.57	.450	2.25
375	.535	2.67	.525	2.62	.456	2.28
380	.530	2.65	.522	2.61	.459	2.28
390	.490	2.45	.485	2.42	.425	2.12
400	.430	2.15	.425	2.12	.376	1.88

Spectra of 4-aminoazobenzene
and its complexes

Beckman Spectrophotometer Model DU

Temp. 25° C. pH 6.8

Wavelength m.micron	Dye: Optical Density	Dye: Extention Coefficient x 10^4	Dye-Normal complex: Optical Density	Dye-Normal complex: Extention Coefficient x 10^4	Dye-Tumor complex: Optical Density	Dye-Tumor complex: Extention Coefficient x 10^4
350	.270	1.35	.245	1.22	.266	1.33
360	.323	1.61	.300	1.50	.322	1.61
370	.352	1.76	.330	1.65	.350	1.75
375	.356	1.78	.335	1.67	.353	1.76
380	.352	1.76	.332	1.66	.350	1.75
390	.325	1.62	.308	1.54	.324	1.62
400	.285	1.42	.268	1.34	.287	1.43

Normal liver homogenate and 4'-methyl-4-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-6})	AMT. BOUND M (10^{-8})	MG. PROTEIN	%BOUND	DEV.	$DEV.^2$
1	1.43						
1p	1.26	.17	.425	10	11.90	.5	.25
2	1.96						
2p	1.68	.28	.700	10	14.30	2.9	8.41
3	4.81						
3p	4.13	.68	1.700	10	14.10	2.6	6.76
4	1.68						
4p	1.45	.23	.575	10	13.70	10.7	114.49
5	2.04						
5p	1.59	.45	1.120	10	22.10	.4	.16
6	5.00						
6p	3.95	1.05	2.620	10	21.00	9.3	86.49
7	1.36						
7p	1.21	.15	.375	10	11.00	4.4	19.36
8	1.90						
8p	1.86	.04	.100	10	2.11	5.8	33.64
9	4.75						
9p	4.20	.55	1.375	10	8.60	2.8	7.84
10	2.50						
10p	2.10	.40	1.000	10	16.00	4.4	19.36
11	4.22						
11p	3.90	.32	.800	10	7.60	3.8	14.44
12	6.45						
12p	5.57	.88	2.200	10	13.60	2.2	4.84
13	2.50						
13p	2.36	.14	.350	10	5.60	5.8	33.64
14	3.91						
14p	3.61	.30	.750	10	7.60	3.8	14.44
15	6.00						
15p	5.40	.60	1.500	10	10.00	1.4	1.96

Normal liver homogenate and Beckmen Spectrophotometer Model Du
4'-methyl-4-aminoazobenzene (concl.)

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-6})	AMT. BOUND M (10^{-8})	MG. PROTEIN	% BOUND	DEV.	$DEV.^2$
16	2.50						
16p	2.37	.13	.325	10	5.2	6.2	38.44
17	3.91						
17p	3.65	.26	.625	10	6.6	4.8	23.04
18	6.00						
18p	5.31	.69	1.725	10	11.5	.1	.01
19	2.76						
19p	2.51	.25	.625	10	9.0	2.4	5.76
20	4.18						
20p	3.56	.62	1.550	10	14.8	3.4	11.56
21	6.54						
21p	5.38	1.16	2.900	10	16.8	5.4	29.16
22	5.55						
22p	5.00	.55	1.370	10	9.8	1.6	2.56
23	5.35						
23p	4.82	.53	1.330	10	9.8	1.6	2.56
24	5.45						
24p	4.82	.63	1.570	10	11.6	.2	.04

Tumor liver homogenate and 4'-methyl-4-aminoazobenzene — Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	ΔCONC. (10^{-8})	AMT.BOUND: M (10^{-8})	MG. PROTEIN	% BOUND	DEV.	DEV.^{2}
1	3.00						
1p	2.30	.70	1.750	10	23.2	9.55	91.20
2	3.81						
2p	3.20	.61	1.530	10	15.8	2.15	4.60
3	5.72						
3p	5.00	.72	1.800	10	12.5	1.15	1.32
4	2.41						
4p	1.95	.46	1.150	10	19.1	5.45	29.70
5	3.59						
5p	3.09	.50	1.250	10	14.0	.35	.12
6	5.60						
6p	5.00	.60	1.500	10	10.7	2.95	8.70
7	3.38						
7p	3.14	.24	.600	10	7.1	6.55	42.90
8	5.18						
8p	4.47	.71	1.780	10	13.7	.15	.02
9	2.18						
9p	1.97	.21	.525	10	9.6	4.05	16.40
10	3.26						
10p	2.98	.28	.700	10	8.6	5.05	25.02
11	5.12						
11p	4.69	.43	1.075	10	8.4	4.85	23.52
12	2.23						
12p	1.80	.43	1.075	10	19.2	5.55	30.80
13	3.27						
13p	2.96	.31	.775	10	10.5	3.15	9.92
14	5.00						
14p	4.60	.40	1.000	10	8.0	5.65	31.97
15	3.30						
15p	2.96	.34	.850	10	10.3	3.35	11.22

Tumor liver homogenate and Beckman Spectrophotometer Model DU
4'-methyl-4-aminoazobenzene (concl.)

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-6})	AMT. BOUND M (10^{-8})	MG. PROTEIN	% BOUND	DEV.	DEV.^{2}
16	3.38						
16p	2.88	.50	1.250	10	14.8	1.15	1.32
17	1.86						
17p	1.43	.38	.950	10	20.5	6.85	46.92
18	1.73						
18p	1.45	.28	.700	10	16.2	2.55	6.50
19	1.75						
19p	1.62	.13	.330	10	7.4	5.85	34.22
20	1.75						
20p	1.45	.30	.750	10	17.1	3.45	11.90
21	1.77						
21p	1.47	.30	.750	10	16.9	3.25	10.56
22	1.77						
22p	1.47	.30	.750	10	16.9	3.25	10.56

Normal liver homogenate and 3'-methyl-4-aminoazobenzene — Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. :M/L(10^{-6})	Δ CONC. (10^{-6})	AMT. BOUND: M (10^{-6})	MG. PROTEIN	% BOUND	DEV.	$DEV.^2$
1	2.37						
1p	1.99	.38	.950	10	16.00	8.50	72.25
2	4.20						
2p	3.66	.54	1.350	10	12.90	5.40	29.16
3	6.14						
3p	5.08	1.06	2.650	10	17.30	9.80	96.04
4	2.34						
4p	2.16	.18	.450	10	7.70	.20	.04
5	4.07						
5p	3.78	.29	.724	10	7.15	.35	.12
6	5.85						
6p	5.39	.46	1.150	10	7.85	.35	.12
7	2.38						
7p	2.04	.34	.850	10	14.30	6.80	46.24
8	4.11						
8p	3.58	.53	1.320	10	12.90	5.40	29.16
9	5.94						
9p	5.22	.72	1.800	10	12.95	5.45	29.70
10	2.44						
10p	2.20	.24	.600	10	9.80	2.30	5.29
11	4.85						
11p	4.22	.36	.900	10	7.85	.35	.12
12	6.01						
12p	5.36	.65	1.620	10	10.70	3.20	10.24
13	4.15						
13p	3.79	.36	.900	10	8.69	1.19	1.41
14	2.64						
14p	2.38	.26	.650	10	9.85	2.35	5.52
15	1.63						
15p	1.50	.13	.325	10	7.98	.48	.23

Normal liver homogenate and Beckman Spectrophotometer Model DU
3'-methyl-4-aminoazobenzene (concl.)

Temp. 0° C. pH 6.8

TUBE: #	CONC. :M/L(10^{-6})	Δ CONC.: (10^{-6})	ΔT.BOUND: M (10^{-8})	MG. : PROTEIN	% BOUND	DEV.	$DEV.^2$
16	3.98						
16p	3.68	.30	.750	10	7.50	.00	.00
17	3.98						
17p	3.80	.18	.450	10	4.75	2.75	7.56
18	3.96						
18p	3.66	.30	.750	10	7.60	.10	.01
19	5.62						
19p	5.15	.47	1.170	10	8.35	.85	.72
20	5.59						
20p	5.23	.36	.900	10	6.45	1.05	1.10

Tumor liver homogenate and 3'-methyl-4-aminoazobenzene

Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-6})	AMT. BOUND M (10^{-8})	MG. PROTEIN	% BOUND	DEV.	DEV.^{2}
1	1.86						
1p	1.64	.23	.575	10	12.30	3.3	10.89
2	2.37						
2p	1.99	.38	.950	10	11.00	2.1	4.41
3	.94						
3p	.80	.14	.350	10	14.90	6.0	36.00
4	1.76						
4p	1.58	.18	.450	10	10.20	1.3	1.69
5	2.76						
5p	2.50	.26	.650	10	9.40	.5	.25
6	3.72						
6p	3.37	.35	.875	10	9.40	.5	.25
7	4.78						
7p	4.38	.40	1.000	10	8.40	.5	.25
8	.90						
8p	.78	.12	.300	10	13.30	4.4	19.36
9	1.79						
9p	1.68	.11	.275	10	6.30	2.6	6.76
10	2.84						
10p	2.65	.19	.475	10	6.70	2.2	4.84
11	3.76						
11p	3.40	.36	.900	10	9.60	.7	.49
12	4.90						
12p	4.43	.47	1.170	10	9.60	.7	.49
13	.92						
13p	.79	.13	.325	10	14.10	5.2	27.04
14	3.79						
14p	3.43	.36	.900	10	9.50	.6	.36
15	3.86						
15p	3.57	.29	.725	10	7.50	1.4	1.96

Tumor liver homogenate and 3'-methyl-4-aminoazobenzene (concl.)

Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-6})	AMT. BOUND: M (10^{-8})	MG. PROTEIN	% BOUND	DEV.	DEV.^{2}
16	4.96						
16p	4.57	.39	.975	10	7.90	1.0	1.00
17	5.42						
17p	5.10	.32	.800	10	5.90	3.0	9.00
18	6.00						
18p	6.00	.00	.000	10	0.00	0.0	0.00
19	6.60						
19p	6.00	.60	1.500	10	9.10	.2	.04
20	6.55						
20p	6.05	.50	1.250	10	7.60	1.3	1.69
21	5.62						
21p	5.15	.47	1.170	10	8.40	.5	.25
22	5.35						
22p	5.15	.20	.500	10	3.70	5.2	27.04
23	6.55						
23p	6.05	.49	1.230	10	7.50	1.4	1.96

Normal liver homogenate and 4-aminoazobenzene — Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-8})	AMT. BOUND: M (10^{-8})	MG. PROTEIN	%BOUND	DEV.	DEV.^{2}
1	4.59						
1p	4.52	.07	.175	10	1.53	3.25	10.56
2	6.51						
2p	6.41	.10	.250	10	1.53	3.25	10.56
3	2.68						
3p	2.54	.14	.350	10	5.20	.42	.17
4	4.69						
4p	4.38	.31	.780	10	6.60	1.82	3.31
5	6.58						
5p	6.35	.23	.575	10	3.50	1.28	1.63
6	2.43						
6p	2.29	.14	.350	10	5.75	.97	.94
7	3.86						
7p	3.59	.27	.695	10	6.98	2.20	4.84
8	4.78						
8p	4.42	.36	.400	10	7.50	2.72	7.39
9	3.76						
9p	3.64	.12	.300	10	3.20	1.58	2.49
10	4.68						
10p	4.42	.26	.650	10	5.55	.77	.59
11	2.40						
11p	2.31	.09	.230	10	3.75	1.03	1.06
12	3.94						
12p	3.72	.22	.550	10	5.60	.82	.67
13	4.76						
13p	4.66	.10	.250	10	2.10	2.68	7.18
14	3.84						
14p	3.68	.16	.400	10	4.17	.61	.37
15	3.88						
15p	3.71	.17	.425	10	4.37	.41	.16

Normal liver homogenate and 4-aminoazobenzene (concl.)

Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-8})	AMT. BOUND M (10^{-8})	MG. PROTEIN	% BOUND	DEV.	$DEV.^2$
16	5.43						
16p	5.25	.18	.450	10	3.30	1.48	2.19
17	5.49						
17p	5.05	.44	1.100	10	8.00	3.22	10.36
18	5.35						
18p	4.95	.40	1.000	10	7.50	2.72	7.39

Tumor liver homogenate and 4-aminoazobenzene — Beckman Spectrophotometer Model DU

Temp. 0° C. pH 6.8

TUBE #	CONC. M/L(10^{-6})	Δ CONC. (10^{-8})	AMT. BOUND M (10^{-8})	MG. PROTEIN	% BOUND	DEV.	DEV.^{2}
1	3.40						
1p	3.15	.25	.625	10	7.35	2.30	5.29
2	3.67						
2p	3.34	.33	.825	10	9.00	3.95	15.60
3	5.14						
3p	5.00	.14	.350	10	2.72	2.33	5.42
4	6.85						
4p	6.60	.25	.625	10	3.65	1.40	1.96
5	8.54						
5p	8.15	.39	.975	10	4.55	.50	.25
6	3.30						
6p	3.19	.11	.275	10	3.33	1.72	2.95
7	3.59						
7p	3.35	.24	.600	10	6.70	1.65	2.72
8	3.30						
8p	3.12	.18	.450	10	5.45	.40	.16
9	3.63						
9p	3.24	.39	.975	10	10.70	5.65	31.92
10	5.05						
10p	4.92	.13	.325	10	2.57	2.48	6.15
11	6.80						
11p	6.40	.40	1.000	10	5.90	.85	.72
12	8.35						
12p	8.11	.24	.600	10	2.87	2.18	4.75
13	6.80						
13p	6.58	.23	.550	10	3.38	1.67	2.78
14	8.35						
14p	8.14	.21	.525	10	2.52	2.53	6.40

INTERACTION OF AZO DYES AND RAT LIVER PREPARATIONS

by

ROBERT M. GROSSMAN

B. S., The University of Nebraska, 1950

AN ABSTRACT OF THESIS

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Listed among the carcinogenic agents, of the oncologist, are certain aminoazo dyes. These compounds have become useful instruments for the study of the interaction of carcinogenic agents and tumor formation. Although the extent of the reaction and the type of tumor resulting may not be the same for each chemical compound, it seems reasonable to assume that essentially the same biochemical changes will take place regardless of the specific character of the chemical compound. On the basis of such a hypotheses a study has been made of the homologs of the carcinogen 4-dimethylaminoazobenzene and methyl orange. This study gave a comparison of the rate of uptake of each homolog of DAB with tumor and normal liver and also showed the constituent of the rat liver that was doing the binding.

Young, mature, albino Sprague-Dawley rats, 180-200 grams in weight were fed 0.06 per cent of 3'-Me-DAB for fourteen weeks. The average amount of ration consumed daily, per rat, was about 15.0 grams.

At the end of fourteen weeks, the animals were anesthetized with ether, the livers perfused with 0.89 per cent saline and excised. The livers were stored for a week in the freezing compartment of a refrigerator. Normal and tumor livers were homogenized and dried as well as fractionated into albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein. The fractionations were made on the basis of differential solubility in solutions of sodium chloride and ammonium sulfate.

Spectral changes were used to indicate qualitative binding and the dialysis equilibrium method was used for a more

quantitative determination of the degree of binding.

The solubility of the two liver homogenates were determined. The tumor liver homogenate was found to be slightly more soluble in phosphate buffer (pH 6.8) than the normal liver homogenate.

The per cent of protein, glycogen and lipid was determined in the water soluble fraction of the two homogenates. The amount of glycogen and lipid in the two fraction was insignificant when compared to the amount of protein. The tumor liver homogenate contained 71.76 per cent protein and the normal liver homogenate contained 56.85 per cent protein.

Examination of the normal and tumor fractions, albumin, globulin, ribonucleoprotein and desoxyribonucleoprotein, showed that they did not form complexes with methyl orange, an anionic dye, or with 4-aminoazobenzene, an uncharged dye.

The normal and tumor liver homogenates did not form complexes with methyl orange. The normal and tumor liver homogenates did form complexes with the uncharged azo dyes. 4'-Methyl-4-aminoazobenzene, the homolog of the carcinogen 4'-methyl-4-dimethylaminoazobenzene, the least soluble dye and the most basic dye reacted to the greatest extent with the normal (10.32% of dye bound) liver homogenate and tumor (13.08% of dye bound) liver homogenate. 3'-Methyl-4-aminoazobenzene, the homolog of the carcinogen 3'-methyl-4-dimethylaminoazobenzene, then complexed 6.79 per cent with the normal liver homogenate and 8.55 per cent with the tumor liver homogenate. 4-Aminoazobenzene, the homolog of 4-dimethylaminoazobenzene, the most soluble dye and the least basic dye complexed the least with

the two homogenates; 4.33 per cent with the normal liver homogenate and 4.84 per cent with the tumor liver homogenate.

It has been shown that the protein fraction of the two homogenates is the fraction that is responsible for the formation of dye complexes. The dye-protein complex could be made through hydrogen bonding or perhaps through van der Waals forces. The exact mechanism for the complex formation has not been conclusively proven.